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STUDIES OF EPSTEIN-BARR VIRUS INFECTION AND IMMUNOREGULATION IN PATIENTS WITH AIDS AND AIDS-RELATED DISORDERS

FINAL REPORT

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The goal of this research effort was to identify the mechanisms responsible for growth of EBV-immortalized B cells and their immune regulation in vivo. In addition, this research sought to define defects of growth and regulation in EBV-infected B cells during HIV infection. Major findings include: 1) identification of two molecules, IL-6 and lactic acid, responsible for autocrine growth stimulation of EBV-immortalized B cells; 2) recognition of a novel regulatory mechanism responsible responsible for growth regulation of EBV-immortalized B cells where T cells deplete growth factors required by EBV-immortalized B cells for growth and 3) identification of abnormal serum IL-6 concentrations in HIV-infected individuals and demonstration of IL-6 tumorigenicity for EBV-immortalized B cells.

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FOREWORD

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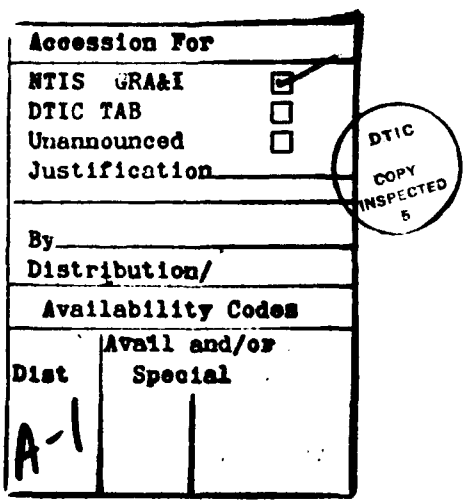
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Introduction

Epstein-Barr virus (EBV) is a herpesvirus that asymptomatically infects the majority of adult individuals worldwide (1). *In vitro*, EBV can infect human B cells and, as a consequence of infection, a proportion of these cells become immortalized into long-term, progressively expanding, cell lines (2). *In vivo*, EBV latently infects a proportion of the circulating B cells, but unlike the situation *in vitro*, these B cells generally fail to progressively proliferate. Rather, the number of circulating B cells remains low and constant overtime, with 1 to 3 B cells per 10^6 circulating B cells being virally infected (3).

Much evidence has emerged to indicate that the control of latent EBV infection in normal EBV seropositive individuals is mainly immunologic (4). In particular, T cell immunity has been found to be solely responsible for this control. Indeed, congenital or acquired T cell severe immunodeficiencies are associated with the development of EBV related malignancies of B lymphocytes (5,6).

The acquired immunodeficiency syndrome (AIDS) is also associated with the development of undifferentiated lymphomas with a high frequency. A recent analysis has established that as many as 50% of AIDS patients receiving long-term AZT treatment go on to develop a B cell malignancy (7).

The present studies were designed to better understand the basis for development of EBV positive malignancies. Three topics were selected for in-depth investigation: 1) study of the mechanisms of T cell control of latent EBV infection in normals and AIDS patients; 2) study of the molecular basis for B cell proliferation/immortalization by EBV; and 3) study of dysregulated growth of EBV-infected B cells.

Body

T cell regulation of latent EBV infection. T lymphocytes are responsible for regulation of latent EBV infection in EBV seropositive normal individuals (for a review, 3). The mechanisms of T cell regulation, however, are still incompletely understood.

In vitro studies have demonstrated that EBV-specific T cell cytotoxicity, natural cytotoxicity, T cell suppression, interferon α and γ production are all effective mechanisms of control for B cells infected with EBV (3). Whether these mechanisms also operate *in vivo* is unknown. Particularly, cytotoxicity would appear to be an unlikely mechanism of T cell control *in vivo* because it would be difficult to reconcile with the existence of a long-lived pool of B cells latently infected with the virus.

B cells infected with EBV *in vivo* are long-lived, rather than an everchanging subset of B cells continuously infected de novo by virus released in the oropharynx (8). It has been shown that treatment with high dose Acylovir is associated with suppression of EBV replication in the oropharyngeal epithelium but not with suppression of latent EBV infection in the B cells, indicating that the two compartments of virus persistence are independently regulated (9). In addition, total body irradiation, which is associated with destruction of the B cell compartment but not the oropharyngeal epithelium, was reported to cause a prolonged absence of EBV-infected B cells in the circulation (10). This suggests that EBV infection in the oropharynx is not responsible for maintenance of latent B cell infection with EBV.

A non-cytotoxic T cell control would appear a more likely mechanism for regulation of EBV-infected B cells. Evidence for this mechanism was

previously obtained in studies *in vitro* (11). However, in the absence of suppressor molecules responsible for the inhibition, suppression remained a poorly defined process. Here we examined alternative mechanisms for suppression. Particularly, we tested whether T cell competition for the growth factors required by EBV-immortalized B cells for autocrine growth might represent a mechanism for T cell inhibition. Our results show that T cells can deprive EBV-infected B cells of the growth factors they need to expand and, as a consequence, can inhibit their growth.

As shown in Fig. 1a, culture supernatants of EBV-immortalized lymphoblastoid cell lines (LCL) induced a dose-dependent increase of proliferation in autologous EBV-immortalized B cells cultured at low cell density. Mean induction of proliferation was 14.5-fold above background at supernatant concentrations of 25% (Fig. 1b). After incubation for 2 hours with autologous T cells preactivated *in vitro* with autologous EBV-infected B cells, the supernatants contained reduced levels of autocrine growth factor activity (Fig. 1a), and stimulated the target B cells only by a mean 6.4-fold above background at a concentration of 25% (Fig. 1b). Similar results were obtained with phytohemagglutinin (PHA)-activated T cells (not shown). It should be noted that simple addition of culture medium incubated with activated T cells had minimal or no effect on the proliferation of indicator resting B cells (not shown), suggesting that secretion of inhibitory molecules is not an explanation for the observed results. Also, 14-day EBV-activated B cells, used as controls for activated T cells in parallel incubations, had little or no capacity to remove autocrine growth factor activity from supernatants of EBV-immortalized B cells.

LCL culture supernatants could also enhance T cell proliferative responses to mitogen or antigen. As shown (Fig. 2a), cell-free LCL supernatants induced a dose-dependent increase of T cell proliferation to PHA (mean induction of 10-fold at 50% supernatant). In the absence of PHA, the supernatants induced little or no T cell proliferation. Enhanced proliferation by LCL supernatants (18.4-fold stimulation at 50% supernatant) was also observed when the T cells were costimulated by autologous irradiated EBV-immortalized B cells (Fig. 2b). Irradiation was used to prevent the EBV-immortalized B cells from proliferating without affecting their viability.

Because activated T lymphocytes could remove autocrine growth factor activity including IL-6 from LCL culture supernatants, we asked whether this might represent a mechanism for T cell control of EBV-induced B cell growth. B lymphocytes infected *in vitro* with EBV become activated into immunoglobulin (Ig) secreting cells and this process is inhibited by autologous EBV-seropositive T cells (Fig. 3). Addition of autologous cell-free LCL culture supernatants to T cell suppressed B cell cultures resulted in reversal of the inhibition (Fig. 3). The degree of reversal was proportional to the dose of T cells added in culture (Fig. 3a); at a T to B cell ration of 2:1 suppression averaged 95% in medium alone and 41% in medium with supernatant (54% mean reversal of suppression, Fig. 3b). As expected, cell-free supernatants of LCL also stimulated EBV-infected B cells cultured alone. This stimulatory effect, however, was significantly lower than that observed in mixed B and T cell cultures ($p < 0.01$), so that reversal of suppression in this system could not be attributed solely to B cell stimulation by autocrine growth factors.

Together, these experiments indicate that T cell competition for growth factors required by EBV-infected B cells to proliferate represents an

effective *in vitro* mechanism for T cell regulation of B cell growth induced by EBV. We suggest that this ability of T cells to deplete EBV-infected B cells of the growth factors they require to proliferate could be central to their *in vivo* control of EBV latency in B cells.

T cell recognition *in vivo* of latent EBV peptides in conjunction with B cell histocompatibility antigens (12,13) could result in T cell clonal expansion in the proximity of EBV-infected B cells resulting in local depletion of the growth factors required by the virally infected B cells to proliferate. The effect of such a process would be to continuously limit the growth of B cells latently infected with EBV while permitting the long-lasting presence of a small population of EBV-infected B cells in the circulation. Thus, T cell depletion of growth factors required by the EBV-infected B cells to proliferate represents a newly identified *in vitro* mechanism of control which could be essential for *in vivo* maintenance of EBV latency in B lymphocytes.

Growth factor requirements for proliferation of EBV-infected B cells.

It has been observed that the vigorous proliferation of EBV-immortalized B cells cultured at low cell density is dependent upon growth factors found in culture supernatants of either EBV-immortalized B cells or activated monocytes (14-17). Such findings have suggested that autocrine and/or paracrine growth factors may have a role in the maintenance of an immortalized state by this virus. In the case of monocyte supernatants, interleukin-6 (IL-6), a multifunctional cytokine produced in a variety of cell types, was recently identified as being the molecule responsible for the stimulation of growth of EBV-immortalized B cells (17,18). Thus, IL-6 can act as a paracrine growth factor for EBV-immortalized cells.

The molecular nature of the factor(s) responsible for autocrine growth stimulation of EBV-immortalized B cells is still controversial. 3B6 IL-1, a structurally novel protein with IL-1 bioactivity, was reported to be an autocrine growth factor for EBV-immortalized B cells (19,20). Recently, 3B6 IL-1 was found to belong to a family of reducing enzymes known as thioredoxin (21). Recombinant 3B6 IL-1, however, while exhibiting thioredoxin activity, lacked a number of the biological activities attributed to 3B6 IL-1, including IL-1 activity (Bertoglia et al., personal communication). Thus, it was proposed that 3B6 IL-1 might not be an autocrine growth factor for EBV-immortalized B cells, but a copurified contaminant. More recently, affinity-purified, soluble CD23, a B-cell activation antigen expressed at high levels in EBV-immortalized cells that also functions as a low-affinity receptor for immunoglobulin E (IgE), was reported to promote growth in EBV-immortalized B cells and in anti-IgM-stimulated B cells (22). In other studies, however, soluble CD23, while preserving a variety of biological activities attributed to CD23, failed to induce growth in IgM-costimulated B cells (23) and in EBV-immortalized B cells (Tosato et al., unpublished results). Thus, while there is evidence suggesting that 3B6 IL-1 and soluble CD23 might be autocrine growth factors for EBV-immortalized B cells, additional studies will be required to clarify some of the controversial issues.

Our studies provide evidence for the existence of two autocrine growth factor activities produced by EBV-immortalized B cells, distinguishable by size fractionation and the spectrum of biological activities. Much of the autocrine growth factor activity in lymphoblastoid cell line supernatants resided in a low-molecular-weight (<5,000) fraction (24). The molecule responsible for this activity was identified as lactic acid. In addition, up

to 20 to 30% of the autocrine growth factor activity resided in the high-molecular-weight (>5,000) fraction. This high-molecular-weight growth factor activity was attributed to IL-6.

a) **Size heterogeneity of autocrine growth factors for EBV-immortalized B cells.** To maximize autocrine growth factor production, exponentially growing EBV-immortalized cells that had been extensively washed in RPMI 1640 medium were incubated for 24 h at a cell density of 1.5×10^6 cells per ml in RPMI 1640 medium containing 1 mg of BSA per ml and 2.5 μg of transferrin per ml. Autocrine growth factor activity in these supernatants was assessed by a 3-day culture of lymphoblastoid cells that had been starved by incubation for 24 h at a cell density of 5×10^5/ml in RPMI 1640 medium supplemented with 1 mg of BSA per ml. An example of autocrine growth factor production under these conditions is shown in Fig. 4. As shown, the addition of increasing amounts of autologous supernatant resulted in increased levels of [^{3}H]thymidine incorporation by the starved B cells, up to 27-fold over background when 50% of the culture medium consisted of the supernatant of autologous cells prepared as described above. Increases in [^{3}H]thymidine incorporation were accompanied by comparable increases in cell numbers.

Supernatants of the lymphoblastoid cell line VDS-0, prepared in this manner, were size fractionated by ultrafiltration through a membrane with a 5,000-molecular-weight cutoff (YM5; Amicon). As shown in a representative experiment of five performed, most of the autocrine growth factor activity was recovered in the

ultrafiltrate, a fraction containing predominantly molecules with molecular weights lower than 5,000 (Fig. 5). However, a proportion (20 to 30%) of the autocrine growth factor activity was found in the concentrate, a fraction containing predominantly molecules with molecular weights higher than 5,000. The biological activity measured in each of these supernatant fractions was due to conditioning of the culture medium by the lymphoblastoid cells, because control medium incubated without the cells and then fractionated by ultrafiltration (YM5; Amicon) consistently failed to promote growth in EBV-induced lymphoblastoid cells.

To estimate the relative molecular weights of these autocrine growth factor activities, concentrate and ultrafiltrate of the VDS-0 cell line, obtained by size fractionation over a membrane with a 5,000-molecular-weight cutoff, were individually subjected to gel filtration chromatography. When the concentrate of VDS-0 cell line supernatant was fractionated by using a precalibrated Sephadex G-75 column, the autocrine growth factor activity eluted with a relative molecular weight higher than 25,000 (Fig. 6). When the filtrate of the same supernatant was fractionated with a precalibrated Bio-Gel P-2 polyacrylamide column, the autocrine growth factor activity eluted with a relative molecular weight lower than 1,200 (Fig. 6). These findings suggested that supernatants of the EBV-induced lymphoblastoid cell line VDS-0 contained at least two autocrine growth factor activities that differed markedly in size.

b) **Supernatants of EBV-immortalized B cells display IL-6-like activity.** We have previously shown that monocyte-derived as well as *E. coli*-derived IL-6 promotes growth in EBV-immortalized B cells (27,30). We next tested whether IL-6 was present in the supernatant of the EBV-immortalized B cells and whether it could act as an autocrine growth factor for these cells. The IL-6-dependent hybridoma cell line B9 proliferated in response to both the unfractionated supernatant of the VDS-0 cell line and the ultrafiltered concentrate containing predominantly molecules of molecular weights higher than 5,000 (Fig. 7). In contrast, the ultrafiltrate, which contained predominantly molecules of molecular weight lower than 5,000, failed to promote growth of B9 cells despite the fact that this fraction contained autocrine growth factor activity. In the example shown, the unfractionated supernatant of the VDS-0 cell line contained approximately 80 B9 growth factor units per ml of supernatant compared with a standard IL-6 preparation. Supernatants from seven additional EBV-induced lymphoblastoid cell lines were examined in the same manner. All unfractionated supernatants had variable contents of IL-6 activity (assayed on B9 cells), ranging from 0.5 to 17.5 U/ml (Table 1). Similarly, following ultrafiltration, each of the concentrates (containing predominantly molecules of molecular weights higher than 5,000) induced growth in B9 cells. In contrast, all supernatant filtrates (containing mostly molecules of molecular weights lower than 5,000) demonstrated no IL-6 activity (Table 1).

It should be noted that unfractionated supernatants of each of these seven lymphoblastoid cell lines contained autocrine growth factor activity (not shown). When fractionated by ultrafiltration (YM5 filter; Amicon), each of these supernatants demonstrated autocrine growth factor activity both in the high- (>5,000) and in the low (<5,000)-molecular-weight fractions, with a distribution that was comparable to that of the VDS-0 cell line (not shown). Together, these findings suggested that culture supernatants of EBV-immortalized cell lines contain both a high- and a low-molecular-weight autocrine growth factor activity as well as IL-6-like activity.

c. **IL-6 is produced by EBV-immortalized B cells and acts as an autocrine growth factor for these cells.** To assess whether IL-6 is responsible for the autocrine activity in the ultrafiltrate concentrate, neutralization experiments were performed using a mouse monoclonal antibody to human IL-6 (25). This antibody neutralized the autocrine growth factor activity of VDS-0 cell line supernatant that had been fractionated to contain predominantly molecules with molecular weights higher than 5,000 (Fig. 8). In contrast, this monoclonal antibody failed to neutralize autocrine growth factor activity in ultrafiltrates (<5,000 molecular weight) of VDS-0 cell line supernatants (not shown). These findings suggested that IL-6 was present in a size-fractionated (>5,000-molecular-weight) culture supernatant of the VDS-0 cell line and that IL-6 was responsible for the autocrine stimulation of B cells by this size-fractionated supernatant. IL-

6, however, appeared not to be involved in autocrine growth stimulation by culture supernatants size fractionated to contain predominantly molecules with molecular weights lower than 5,000.

d. **Purification of a low molecular weight B cell stimulatory factor from supernatants of lymphoblastoid cell lines and its identification with lactic acid.** Starting material for this purification was the culture supernatant of a lymphoblastoid cell line (VD) cultured for 24 hours at a cell density of 0.5×10^6 cells/ml in a serum-free culture medium consisting of RPMI 1640 supplemented with BSA (1 mg/ml) and transferrin (2.5 μg/ml). Briefly, the purification was accomplished by sequential filtration over an Amicon YM5 (5000 mol wt cutoff) membrane, lyophilization of the filtrate, size fractionation over a P2 gel filtration column, anion exchange chromatography over QAE-Sephadex A-25, filtration over Bio-Sil A Silica gel and SM-2 Biobeads, reversed-phase HPLC over a Synchropak C-18 column and a polyhydroxyethil HILIC HPLC column. Recovery of activity at each purification step was monitored by testing the proliferation of the indicator lymphoblastoid cell line VD. As noted above, this line was also used as a source of the growth factor activity for purification.

The purified material was analyzed by mass spectrometry. Two major components were identified, ossalic acid and lactic acid. Both molecules purchased as chemicals were tested for B cell growth factor activity over a wide range of concentrations. Oxalic acid was found to be inactive. In contrast, lactic acid

stimulated the growth of the indicator VD cells at concentrations ranging between 10 and 1 mM (Table 2). The level of stimulation was comparable to that induced by LCL culture supernatants filtered through a 5000 mol wt Amicon membrane. The concentration of lactic acid in the culture supernatant of EBV-immortalized B cells prepared as described above was found to range between 5 and 8 mM. Thus, lactic acid is present in culture supernatants of LCL at concentrations found to promote growth in EBV-immortalized B cells. In addition, the specific B cell growth promoting activity of lactic acid present in LCL culture supernatants was found to be comparable to that of 98% pure lactic acid obtained from a different source. Finally, when highly purified from culture supernatants of LCL, lactic acid was found to promote growth in EBV-immortalized B cells. We therefore concluded that lactic acid is secreted by EBV-immortalized B cells and serves as an autostimulatory molecule for the B cells producing it.

IL-6 is tumorigenic in nude mice

Malignancies of EBV-infected B cells such as X-linked lymphoproliferative disease (26), AIDS-associated lymphomas (27), and post-transplant lymphomas (28) represent unique model systems for which a number of pathogenetic steps have been suggested (29). *In vitro*, B lymphocytes can be immortalized by EBV into long-term cell lines. *In vivo*, B cells naturally infected with EBV are long-lived and subjected to immunoregulatory controls to prevent their expansion (30). In the presence of severe immunodeficiency, EBV-infected B cells can expand *in vivo* giving rise to polyclonal or oligoclonal malignancies.

Interleukin 6 (IL-6), a multifunctional cytokine produced in monocytes, fibroblasts and other cell types (for a review, 31) has been implicated in the pathogenesis of several human cancers, including Kaposi sarcoma (32), cardiac myxoma (33), Castleman's disease (34), multiple myeloma (35), non-Hodgkin B cell lymphomas (36) and B cell chronic lymphocytic leukemia (37). In some of these malignancies IL-6 has been shown to promote growth in the malignant cells by acting as an autocrine or paracrine growth factor. Recently, IL-6 has been identified as an important factor in the establishment and maintenance of EBV-immortalized B cells (17,18). EBV-immortalized B cells secrete low levels of IL-6 in the culture supernatant (24), express surface receptors for IL-6 (38), and can use this cytokine as an autocrine growth factor (24).

To explore a possible role of IL-6 in the multistep process of B cell tumorigenesis, we have stably expressed the human IL-6 gene into EBV-immortalized B cells and examined the ability of these cells to form tumors in athymic mice. Unlike *in vitro* studies that rely upon the contribution of a limited number of cell types, this *in vivo* approach allows one to register the participation of the diverse cell types that may be affected by IL-6 and may contribute or prevent tumor formation. This is particularly relevant in the case of IL-6 that is emerging as one of the most pleiotropic of cytokines with a broad range of biological activities (for a review, 31). While parenteral administration of the cytokine together with EBV-immortalized cells has the potential of affecting all IL-6 responsive targets, this approach is compromised by the relatively short half life of IL-6 (39), and the potential need to achieve sustained effective levels locally. Accordingly, we have used an alternative strategy of expressing the IL-6 gene in the EBV-immortalized

cells. Using this approach, high concentrations of IL-6 may be achieved within the local environment during the entire tumor-immune effector cell interaction. The present experiments show that IL-6 expressing lymphoblastoid cell lines are highly tumorigenic *in vivo*, and this effect is likely due to IL-6 induced dysfunction of natural killer functions. It is hoped that this novel understanding of IL-6 killer cell interactions may provide the basis for therapeutic approaches to cancer treatment.

a. **Generation of Lymphoblastoid Cell Lines Expressing High Level IL-6**

Two lymphoblastoid cell lines, VDS-0 and TB were selected for these studies. These cell lines were originally obtained by EBV (B95-8) immortalization of normal peripheral blood B lymphocytes. VDS-0 is monoclonal and does not secrete immunoglobulin, while TB is polyclonal and secretes IgM, IgG and IgA. Because EBV-immortalized B cells have been shown to express low levels of IL-6, we selected lines that most differed in the levels of endogenous IL-6 secreted. VDS-0 produced the highest IL-6 level (80 U/ml) within a panel of 12, and TB produced one of the lowest (1.6 U/ml) (24).

Complementary DNA sequences spanning the entire human IL-6 coding region were cloned into the retroviral vector depicted in Fig. 9 (40,41). The plasmid pZip-NEO-SV(x)-1-IL6 was transfected into the amphotropic retroviral packaging cell line PA317.3 (), and stably transfected cells were selected as a source of IL-6-encoding virus. Virus containing supernatants from these cells were used to infect the VDS-0 and TB lymphoblastoid cell lines. Five neomycin resistant IL-6 producing cell clones were derived. Their ability to secrete IL-6 in the culture supernatant was assessed in a standard *in vitro* bioassay for IL-6, using the IL-6 dependent hybridoma cell line B9 (43). As shown in Table 3, while the two parent and vector control-

virus infected (neomycin-resistance gene only) cell lines produced little or no IL-6, high level IL-6 secretion was observed in the IL-6 virus-infected cell lines. All the activity detected by the B9 assay was a reflection of human IL-6 produced by the cell lines because it was completely neutralized by a monoclonal antibody to human IL-6 (not shown).

b. Tumorigenicity of high level IL-6 gene expressing lymphoblastoid cell lines

EBV-immortalized lymphoblastoid cell lines are capable of long-term proliferation in tissue culture but generally fail to generate tumors when injected subcutaneously in athymic mice (44). As shown in Table 4, parental and control virus-infected lymphoblastoid cells only rarely caused tumors in nude mice. Tumors developed in only 6 of 80 animals (7.5%), each inoculated subcutaneously with $10x10^6$ cells in one site, and observed over a 15 week period. In contrast, a high proportion (68%) of the mice inoculated with the IL-6 virus-infected lymphoblastoid cell lines developed tumors in parallel experiments.

Variation in IL-6 production among the different IL-6 virus-infected cell lines allows us to examine a possible correlation between the level of tumorigenicity and level of IL-6 produced by each line. The results of such analysis (Table 5) show that neither tumor incidence nor time of first tumor occurrence, correlated directly with the levels of IL-6 produced by the IL-6 virus-infected lines. The observation that IL-6 virus-infected lines are significantly more tumorigenic than control lymphoblastoid lines together with the observation that the level of IL-6 secreted by these lines fails to correlate with tumorigenicity suggests that there might be a critical level of IL-6 sufficient for high level tumorigenicity. Confirmation of this

supposition awaits testing of lymphoblastoid cell lines producing levels of IL-6 lower than those produced by the present lines.

c. **Mixed tumor transplantation assays demonstrate cell transfer of IL-6-associated tumorigenicity**

To test further whether IL-6 is responsible for increased tumorigenicity in EBV-immortalized cells, mixed tumor transplantation assays were performed. To this end, we simultaneously injected the IL-6 virus-infected lymphoblastoid cells together with control cells at the same site. When the IL-6 virus-infected cells were irradiated with 10,000R prior to inoculation, no increased tumorigenicity in the parent line was observed. None of 12 nude mice coinjected with the irradiated IL-6 virus-infected line VDS-0 1 (5 x 10^6/cells/site) and the non-irradiated parent line TB (5 x 10^6 cells/site) developed a tumor, suggesting that sustained high levels of IL-6 were required. In contrast, when the IL-6 virus-infected cells were not irradiated prior to inoculation, increased tumorigenicity in the coinjected control line emerged. Inoculation of the parent TB cell line (5 x 10^6 cell) together with the IL-6 virus-infected VDS-0 1 line (5 x 10^6 cell) in one subcutaneous site resulted in 6 of 10 animals developing a tumor. Histocompatibility typing of the tumor cells within 2 weeks from removal revealed 4 of these tumors to be composed of a mixed cell population, where both HLA types of TB and VDS-0 line were similarly represented. The two remaining tumors only expressed VDS-0 related histocompatibility antigens. These findings demonstrate that tumorigenicity of IL-6 virus-infected lines can be transferred to other cells, and strongly suggests that IL-6 is responsible for these effects.

d. Potential mechanisms mediating tumorigenicity of EBV-immortalized B cells

Given that IL-6 promotes the growth of activated B cells *in vitro*, including EBV-growth immortalized cells (17,18), the tumorigenic effect of IL-6 could depend upon IL-6 providing a growth advantage to EBV-immortalized cells that would allow them to proliferate subcutaneously in the recipient nude mouse. In a series of *in vitro* studies, we could not demonstrate a growth advantage of IL-6 virus-infected lines over control lines. Rather, all lines secreting high levels of IL-6 proliferated less vigorously than the parental cell lines under either optimal culture conditions (cell density 1-3 x 10^5/ml and culture medium composed of RPMI with 10% fetal calf serum) or under suboptimal culture conditions, including low cell densities (1-5 x 10^4 cells/ml) and/or deficient medium (serum-free culture medium, Opti-MEM) (Fig. 10). The inhibited growth seen in IL-6 virus-infected lines could be attributed to direct inhibition of B cell growth by IL-6 (Tosato et al., manuscript in preparation). Thus, under conditions of high IL-6 availability, lymphoblastoid cells appear not to utilize additional IL-6 for increased autocrine growth. While a direct growth-promoting effect of IL-6 on lymphoblastoid cells remains a possibility to explain increased tumorigenicity *in vivo*, the absence of such an effect *in vitro* argues against this hypothesis.

Since the experiments described above indicated that tumorigenicity of IL-6 expressing lymphoblastoid lines is not readily explained on the basis of a direct effect of IL-6 on the B cells, an alternative possibility is that IL-6 might inhibit host immunity.

Tumor histology indicated the presence of small lymphocytes and macrophages at the periphery of the tumors, such that IL-6 might interfere with the proposed antitumor activity of these cells. To address this possibility, spleen cells from nude mice were cultured for 4 days with or without IL-6 (10^8 U/ml) in the presence of IL-2 (400 U/ml). As shown in Table 6, IL-2-activated spleen cells killed efficiently both the lymphoblastoid cell line VDS-0 and the NK-sensitive YAC cell line. Addition of IL-6 together with IL-2 during preculture resulted in marked reduction of target cell killing at all effector to target ratios tested. Because IL-6, at a dose of 10^8 U/ml, had no effect on VDS-0 and YAC-1 target cell killing by cells already activated *in vitro* with IL-2 (not shown) the present experiments demonstrate that IL-6 interferes with IL-2 activation of killer cells. Effective suppression of IL-2 induced killing required a high cytokine concentration (Fig. 11), greater than 10^8 U/ml.

Together, these observations demonstrate that IL-6, at high concentrations, can suppress markedly the activation of IL-2-induced killer cells *in vitro*, and raises the possibility that IL-6-mediated inhibition of tumor cell killing might also occur *in vivo* in the presence of high local cytokine concentrations.

In summary, we have explored a potential role for IL-6 in B cell lymphomagenesis by expressing the human IL-6 gene in normal B cells immortalized with EBV and injecting the IL-6 expressing B cells into nude mice. The experiments show that high level expression of human IL-6 in EBV-immortalized B cells is associated with high level tumorigenicity in athymic mice. It is unlikely that this is due to a direct effect of IL-6 on the B cell because the IL-6 expressing lymphoblastoid cell lines exhibited no growth

advantage *in vitro* over IL-6 non-expressing cells. Rather, IL-6 associated B cell tumorigenicity in this system is likely to depend upon IL-6 inhibiting host tumor immunity.

Polyclonal and oligoclonal malignancies of EBV-infected B lymphocytes have been reported with increasing frequency in individuals with acquired immunodeficiencies such as AIDS patients (7,27) and post transplant recipients (28). The observation that EBV-immortalized B cells secreting high levels of IL-6 have a propensity for giving rise to tumors in immunocompromised hosts raises the possibility that IL-6 may be a factor in human B cell lymphomagenesis. It is worth noting that, while tumors occurred much more frequently with IL-6 virus-infected lymphoblastoid cell lines, parental cell lines occasionally did give rise to tumors in nude mice, and the cell lines derived from these tumors consistently produced higher levels of human IL-6 than the lines originally injected. The occurrence in the nude mouse of selection for high IL-6 secreting clones, raises the possibility that such process might also operate in immunocompromised individuals.

HIV-1 infected individuals generally display abnormally elevated numbers of EBV-infected cells in the peripheral circulation (45). In addition, these individuals often exhibit abnormally elevated serum IL-6 levels (46,47), possibly through a direct action of the virus (46,48). Both these abnormalities could predispose AIDS patients to the development of EBV-associated malignancies. The expanded pool of EBV-infected cells could provide a greater chance for selection of cell clones producing high levels of IL-6, while IL-6, at high levels, could inhibit natural killer functions locally.

IL-6 induction during HIV infection. It was recently reported that HIV, either live or inactivated, induces IL-6 messenger RNA expression and secretion of IL-6 bioactivity in normal mononuclear cells (48). In addition, it is known that human immunodeficiency virus (HIV)-infected individuals have abnormally elevated serum Ig levels (49), and that a proportion of HIV-infected individuals also display abnormally elevated numbers of Epstein-Barr virus (EBV)-infected B cells in the circulation (45). Studies of IL-6 bioactivity *in vitro* have shown that IL-6 stimulates the proliferation of EBV-infected B cells and promotes Ig secretion in activated B cells (17,18). For these reasons, we have further studied the role of HIV as an inducer of IL-6. In particular, we have examined whether HIV, isolated from infected individuals, induces IL-6 secretion *in vitro*. In addition, we have tested whether HIV-infected individuals have abnormally elevated serum IL-6 levels.

a. *Natural isolates of HIV induce IL-6 secretion in human mononuclear cells.* We have examined whether natural isolates of HIV from infected individuals might induce IL-6 production in culture. To this end, highly purified monocyte preparations (>95% nonspecific esterase positive cells) were cocultured with either normal mononuclear cells or mononuclear cells from HIV-infected individuals. At weekly intervals for 8 weeks, beginning on day 8, culture supernatants were analyzed for evidence of HIV infection. As expected, normal monocytes cocultured with normal mononuclear cells had no evidence of HIV production at any time point, as determined by measure of the HIV-associated antigen, p24, in the culture supernatants. In contrast, using the same assay system, monocyte cultures incubated with mononuclear cells from HIV-

seropositive individuals had evidence of HIV production in 12 of 24 samples tested. When monocyte supernatants were tested for IL-6 bioactivity in a standard growth assay of B9 cells, we found that the levels of IL-6 bioactivity were significantly higher in monocyte supernatants with elevated p24 levels when compared to monocyte supernatants with low p24 levels ($P < .04$ Spearman Rank test). Table 7 shows the results of p24 antigen and IL-6 bioactivity determinations in monocyte culture supernatants obtained on day 84 of incubation or at an earlier time point (geometric mean = 36 days) when p24 levels were found to be greater than 250 pg/mL in 2 consecutive weeks. These findings strongly suggest that HIV, isolated from the blood of HIV-seropositive individuals, induces secretion of IL-6 bioactivity in mononuclear cell cultures.

b. *Serum IL-6 levels are elevated in HIV-seropositive individuals.* Because HIV, isolated from infected individuals, induced IL-6 production in mononuclear cell cultures, we asked whether HIV-infected individuals might have abnormally elevated serum IL-6 levels. To this end, serum samples from 10 normal adults and 40 individuals with HIV infection were tested for the presence of IL-6 as determined by the B9 cell growth assay. As shown in Fig. 12, the mean content of IL-6 in normal serum was 1.6 x/+ 1.2 U/mL (geometric mean x/+ SEM). In contrast, the mean serum content of IL-6 in 15 HIV seropositive individuals with stage (1/2) disease was 25.2 x/+ 1.8 U/mL, and in HIV-seropositive individuals with stage (1/4) disease 46.0 x/+ 1.7 U/mL. Both mean serum IL-6

values are significantly different from normal ($P < .001$). HIV-seropositive individuals with late stage (5/6) disease were found to have a mean serum IL-6 content of 2.7 x/+ 1.6 U/mL, a value not different from normals ($P = 1.0$). These findings strongly suggest that HIV-positive individuals (stages 1 through 4) generally have abnormally high levels of IL-6 in their serum.

c. *Serum levels of IL-6 directly correlate with serum levels of IgG, but not of soluble IL-2 receptor (sIL-2R).* In vitro studies have demonstrated that IL-6 promotes B-cell proliferation and Ig production. HIV-infected individuals often have elevated serum Ig, particularly of the IgG isotype (49). We have now examined whether serum levels of IL-6 in HIV seropositive individuals (stages 1 through 6) might correlate directly with serum levels of IgG in the same individuals. As shown in Fig. 13, in 40 HIV seropositive individuals there was a direct relationship between serum IL-6 levels and serum IgG levels ($r = .74$; $P < .001$).

As well as often having abnormally elevated serum Ig levels, HIV-infected individuals have been reported to frequently have abnormally elevated serum levels of sIL-2R (50). This is believed to reflect an underlying state of T-cell activation during HIV infection. However, unlike Ig, in vitro studies have demonstrated that IL-6 does not promote, either alone or with mitogenic costimuli, IL-2R expression in T cells. We have examined the relationship between the two parameters (serum IL-6 levels and sIL-2R levels) in 19 HIV-seropositive individuals (stages 1/2) and found no direct relationship ($r = .01$; $P > .95$). Thus, IL-6,

known to induce Ig production in vitro, was generally found to be abnormally elevated in sera of those HIV-infected individuals with high Ig levels. In contrast, IL-6, a T-cell costimulant that does not promote IL-2 secretion or IL-2 receptor expression in human T cells, was found to be present at variable concentrations in sera of HIV-seropositive individuals with high serum levels of sIL-2R.

In summary, the present findings indicate that natural isolates of HIV from 12 seropositive individuals promote IL-6 production in normal monocytes. In addition, a proportion of HIV-infected individuals with stage 1 through 4 disease have abnormally elevated serum IL-6 levels. What might be the role of IL-6 in the pathogenesis of HIV disease? In vitro studies have shown that IL-6 is a potent stimulator of proliferation and/or Ig production in activated B cells, including EBV activated B cells (17,18). Individuals with HIV infection often have circulating activated B cells and abnormally elevated serum Ig levels. In addition, a proportion of these individuals display abnormally elevated numbers of EBV-infected B cells in the circulation that might predispose them to the occurrence of EBV-containing B-cell lymphoproliferations. It is tempting to speculate that IL-6, chronically induced by HIV, and perhaps a variety of other stimuli, are responsible for promoting Ig secretion and expanding the pool of B cells latently infected with EBV in HIV-infected individuals.

Conclusions

Our studies have been directed toward a better understanding of B cell growth induced by EBV and how this process is abnormally regulated in HIV-seropositive individuals.

The present results reveal that growth of EBV-immortalized and potentially tumorigenic B lymphocytes is dependent upon B cell secretion of at least two growth promoting molecules, IL-6 and lactic acid. Both molecules are necessary for continuous proliferation of the virally immortalized B cells. When absent or present at too low concentrations the immortalized B cells stop growing and eventually die.

We have examined what might be the regulatory mechanisms responsible for limiting the proliferation of EBV-immortalized B cells in vivo. Confirming the well established observation that T lymphocytes are solely responsible for the control of latent EBV infection in EBV seropositive individuals (9), we have now found that T cells are responsible for depleting EBV-infected B cells of the growth factors they require for autocrine growth. T lymphocytes upon exposure to autologous B cells infected with EBV proliferate, and this proliferation is associated with depletion of growth factors secreted by the B cells and required for their growth. As a result of this process, EBV-infected B cells are growth suppressed and, as a consequence, their number kept low.

As mentioned above, IL-6 is one of the autocrine growth factors produced by EBV-immortalized B cells (24). We have found that T cells can deplete IL-6 from culture supernatants of EBV-immortalized B cells.

What happens if IL-6 is present in abnormally high concentrations? We have found that high level expression of the IL-6 gene in EBV-infected normal B cells is associated with their acquisition of a malignant phenotype.

Lastly, we have examined whether serum IL-6 levels might be high in HIV seropositive individuals. It was previously known that the number of EBV-infected B cells is abnormally high in this patient population and that this is likely due to ineffective T cell regulation (45). It was also previously known that HIV may stimulate IL-6 production by normal monocytes. Thus, there were at least two reasons for IL-6 to be elevated in this group of patients. Indeed, we found that HIV-infected individuals with stage 1 through 4 disease have abnormally elevated serum IL-6 levels. Because IL-6 stimulates growth in EBV-immortalized B cells (17,18), it is tempting to speculate that AIDS patients are predisposed to the development of EBV malignancies, in part, by virtue of their abnormal serum IL-6 levels.

Recent evidence suggests that B cell lymphomas frequently occur in AIDS individuals particularly those who are long-term survivors receiving anti-retroviral therapy (17). It is anticipated that lymphomas will represent the leading cause of death in this patient population. In the absence of effective treatments for these malignancies, it is hoped that a better understanding of the pathogenesis of B cell lymphomas may lead to novel therapeutic approaches.

The studies reported here indicate that treatment with an IL-6 inhibitor might be of value to prevent the occurrence of EBV-related malignancies in AIDS patients.

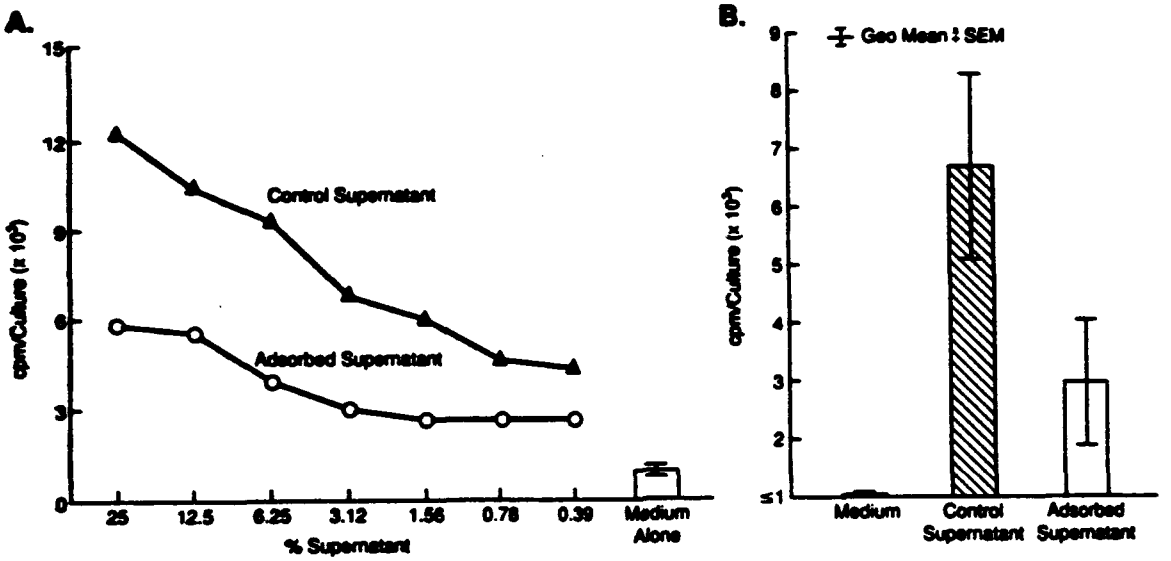


Fig. 1. Activated T lymphocytes adsorb growth factors secreted and used by EBV-immortalized B cells. A) Cell-free culture sypernatants of EBV-immortalized B cells from a normal individual were incubated for 2 hours at 4 °C with autologous T cells that had been preactivated by 14 day culture with autologous EBV-infected B cells or mock incubated. After incubation, cell free supernatants adsorbed or control were tested for residual growth factor activity by culture with autologous EBV-immortalized B cells.

B) Mean results of 5 experiments where supernatants of EBV-immortalized B cells were either mock adsorbed (control supernatant) or adsorbed with activated T cells (adsorbed supernatant) and tested for residual autocrine growth factor activity at a concentration of 25% (v/v).

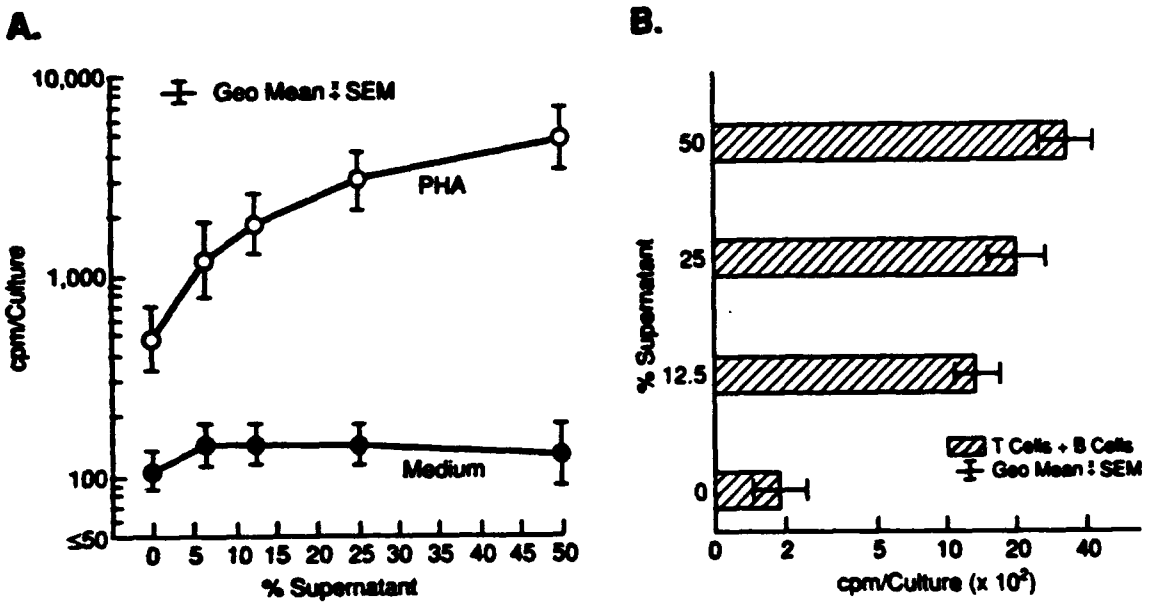


Fig. 2. Supernatants of EBV-immortalized B cells promote T cell proliferation to mitogen and antigen. A) Normal peripheral blood T lymphocytes were cultured with or without PHA with varying amounts of cell free supernatants from autologous EBV-immortalized B cells. Proliferation was measured by ^{3}H-thymidine incorporation after 3 days of culture. The results reflect the mean (x/+ SEM) of 5 determinations. B) EBV-seropositive T cells that had been preactivated by culture with autologous EBV-infected B cells for 8 days were recultured for 5 days with autologous EBV-immortalized, irradiated, B cells with or without cell-free culture supernatant of the autologous EBV-immortalized B cells at the indicated concentrations. Mean of 7 determinations (x/+) SEM.

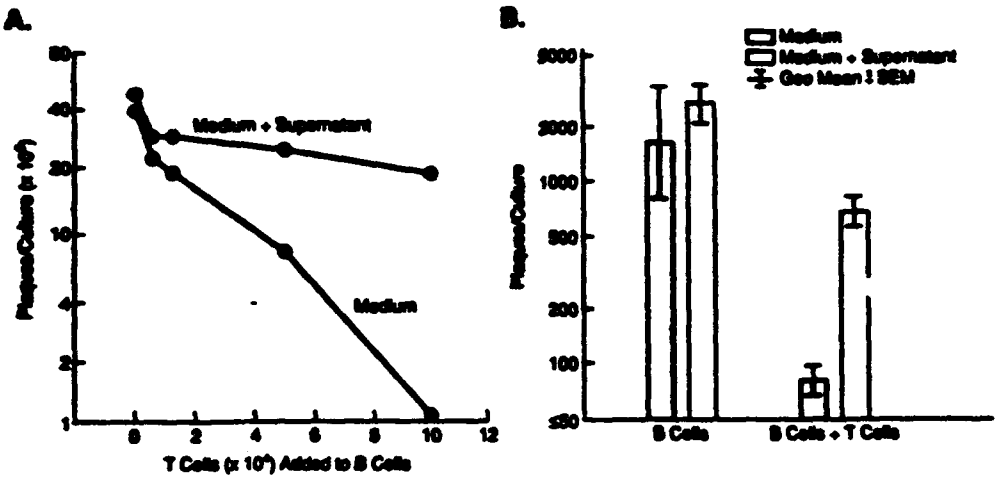


Fig. 3. Reversal of EBV-related T cell suppression by cell-free culture supernatants of autologous EBV-immortalzed B cells.

A) Representative experiment. EBV-infected B lymphocytes ($5x10^5$) from a normal EBV-seropositive individual were cultured either alone or with autologous T cells at the indicated concentrations. Cultures were carried out either in medium alone (medium) or in medium supplemented with cell-free culture supernatant from autologous EBV-immortalized B cells (medium + supernatant). The number of Ig secreting cells was determined at the end of 14 days incubation. B) Mean T cell suppression and reversal of suppression in 5 independent experiments performed as described above.

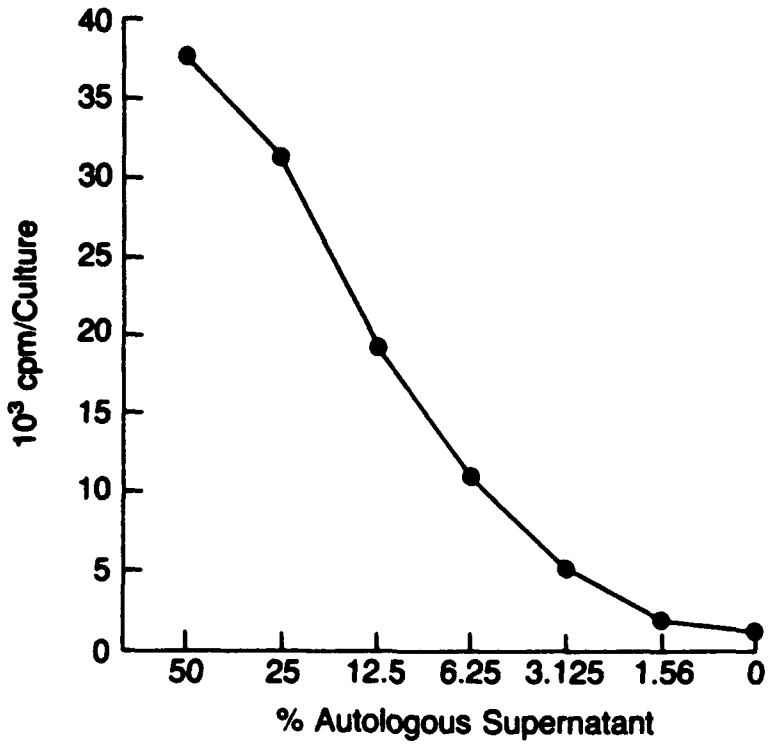


Fig. 4. Autocrine growth stimulation by supernatant of a lymphoblastoid cell line. Starved cells from the EBV-immortalized B-cell line VDS-O (3×10^3 cells per well) were cultured in 0.2 ml of culture medium (RPMI 1640 medium plus 1 mg of BSA per ml and 2.5 μg of transferrin per ml) supplemented with various amounts of autologous culture supernatant (prepared by 24-h culture of VDS-O cells at 1.5×10^6 cells per ml in RPMI 1640 medium with 1 mg of BSA per ml plus 2.5 μg of transferrin per ml).

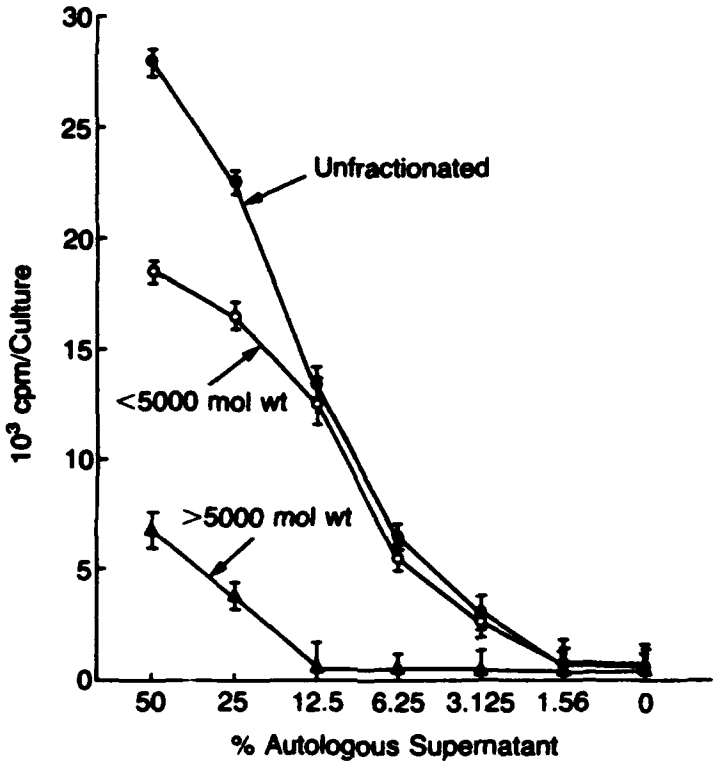


Fig. 5. Size heterogeneity of autocrine growth factor activity produced by EBV-immortalized B cells. Starved cells from the EBV-immortalized cell line VDS-O (3×10^3 cells/well) were cultured in 0.2 ml of culture medium (RPMI 1640 plus 1 mg/ml BSA plus 2.5 μg/ml transferrin) supplemented with various amounts of autologous culture supernatant either unfractionated or size fractionated by Amicon filtration over a YM5 membrane.

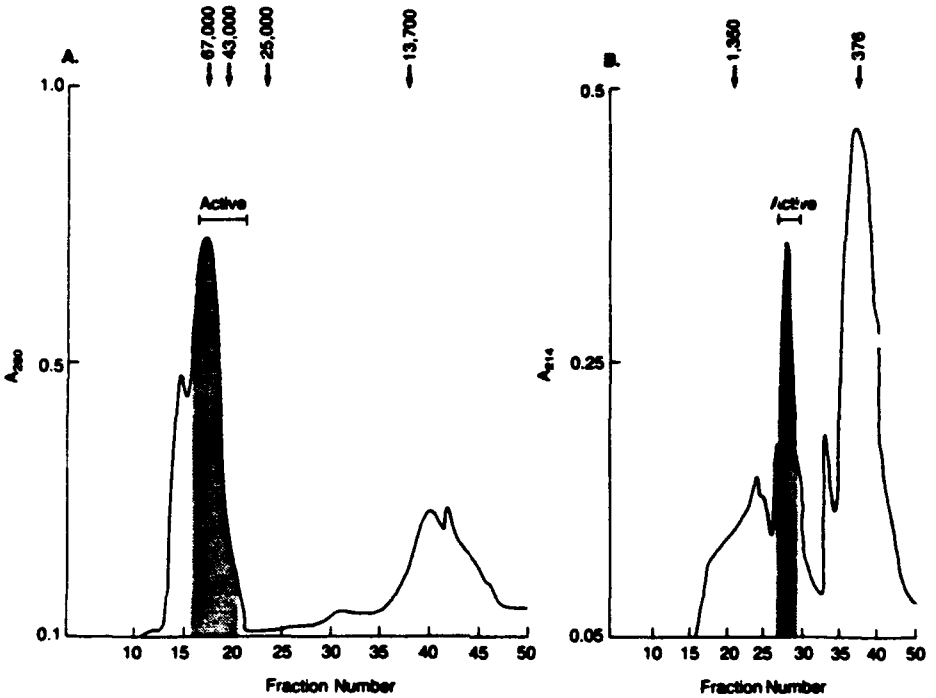


Fig. 6. Gel filtration chromatography of lymphoblastoid cell line-derived autocrine growth factors. (A) Supernatant of the lymphoblastoid cell line VDS-O, prepared as described above, and concentrated 25-fold by ultrafiltration (YM5 filter; Amicon) was applied (3 ml) to a preequilibrated gel filtration column (Sephadex G-75, 1.5 by 75 cm) and eluted in 0.1 M Tris (pH 7.5; flow rate, 10 ml/h). Each fraction was assayed for autocrine growth factor activity. Shaded area denotes the active fractions. Molecular weights are indicated at the top.

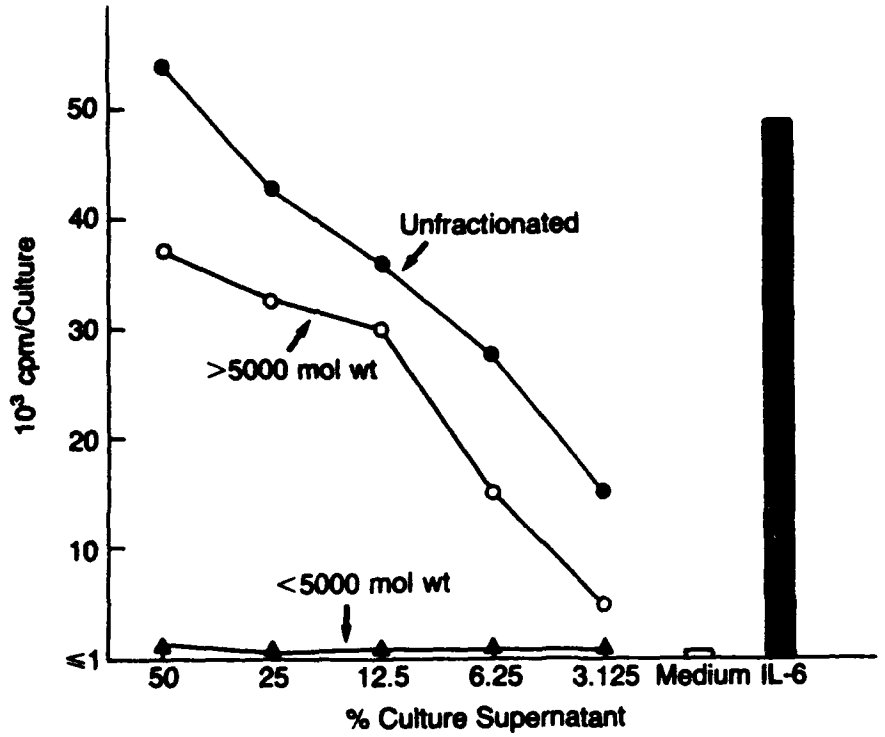


Fig. 7. Supernatant of a lymphoblastoid cell line displays IL-6 activity. Supernatant of the EBV-immortalized cell line VDS-O was tested for IL-6 activity, either as unmanipulated supernatant (unfractionated) or following size fractionation through a YM5 membrane (Amicon) with a 5,000-mol wt cutoff. Recombinant IL-6 (200 ng/ml) was used as a positive control.

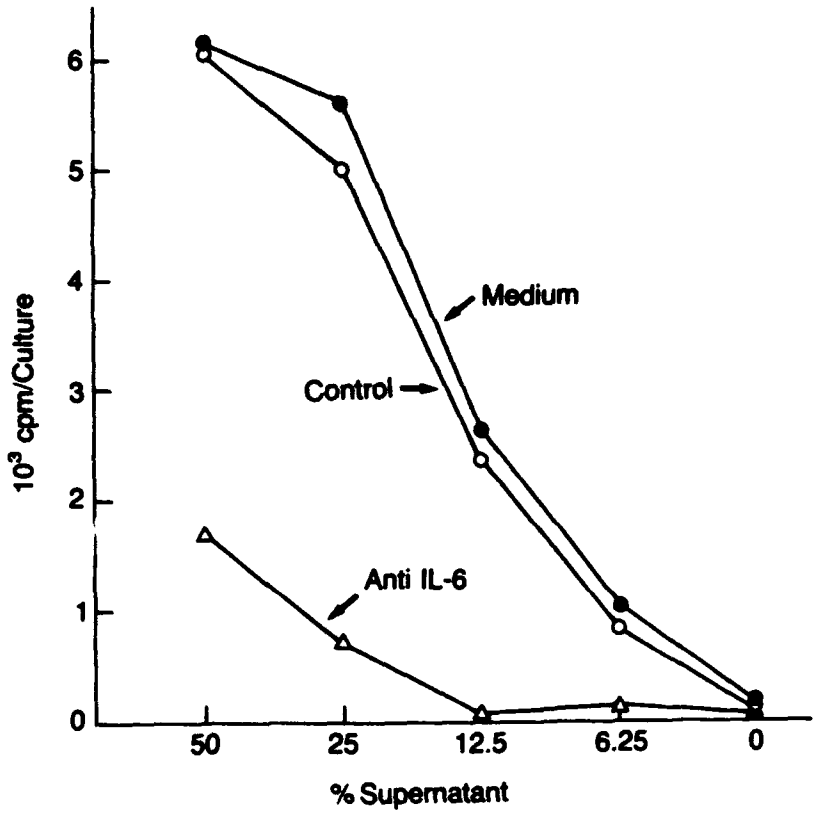


Fig. 8. Autocrine growth factor activity of a lymphoblastoid cell line supernatant is neutralized by a monoclonal antibody to human IL-6. Two-fold concentrated supernatant of the cell line VDS-O, which had been size fractionated to contain mostly molecules with a mol wt > 5000 were incubated for 1 hour at 37 °C with either medium or with a control mouse monoclonal antibody (10 μg/ml) or with a mouse monoclonal antibody to human IL-6 (10 μg/ml). After incubation EBV-immortalized B cells were added and proliferation tested after 3 days of culture.

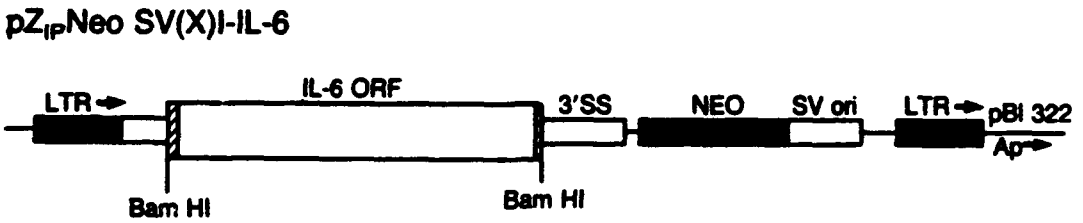


Fig. 9. Diagrammatic representation of the IL-6 retroviral vector used for transfection of the retroviral vector used for transfection of the retroviral packaging cell line PA317. The open reading frame (ORF) for human IL-6, isolated as a Hind III and Bam I fragment (0.67 kb) from the cDNA clone p-Beta-2-22 (May et al., 1986), was inserted at the Bam HI site of the vector pZip-NEO-SV(x)1 (Cepco et al., 1984).

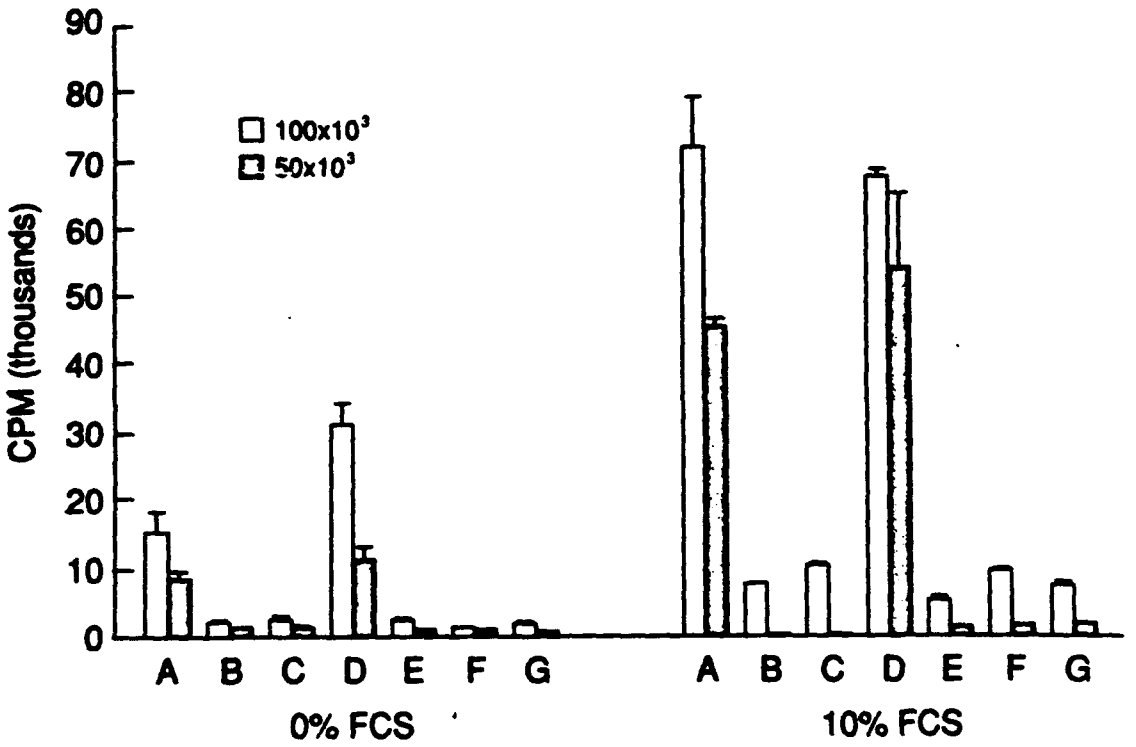


Fig. 10. In vitro proliferative responses of parental TB and VDS-O cell lines and IL-6 expressing TB and VDS-O clones. TB (A), TB 1 (B), TB 5 (C), VDS-O (D), VDS-O 1 (E), VDS-O 2 (F), and VDS-O 3 (G) lines were cultured at 100 x 10^4 (clear bar) and 50 x 10^4 (hatched bar) in either Opti-MEM or RPMI supplemented with 10% fetal calf serum. Cell proliferation was measured by [^{3}H]-thymidine incorporation during the final 4.5 hours of a 3 day incubation. Results are expressed as the mean cpm of triplicate samples ± SEM.

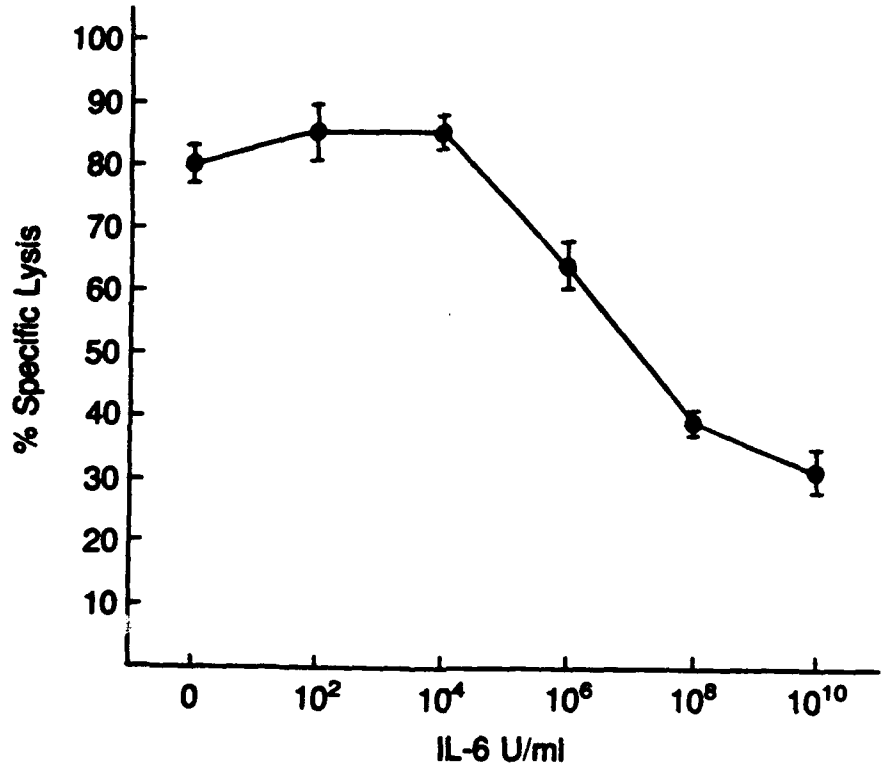


Fig. 11. IL-6 inhibition of IL-2 induced killing is dose-dependent. Combined splenocytes from 3 nude mice were preincubated for 4 days in medium supplemented with either IL-2 alone (500 U/ml) or with IL-2 plus IL-6 at varying concentrations (10^2–10^{10} U/ml). After incubation, the cells were tested in triplicate for cytotoxic activity against ^{51}Cr labeled VDS-O target cells at a ratio of 100:1. The results are expressed as % mean specific lysis (+/- standard deviation).

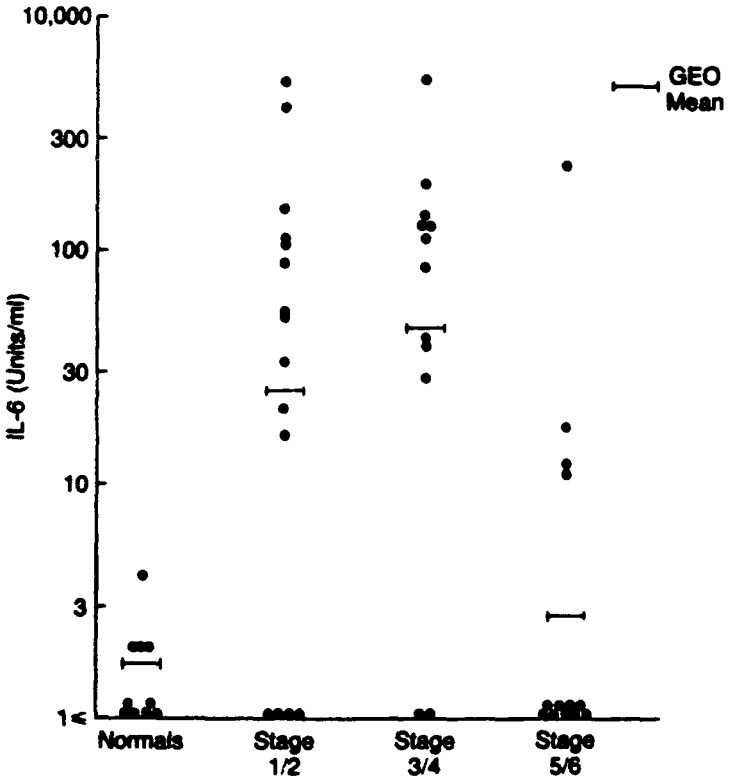


Fig. 12. IL-6 bioactivity content in normal and HIV-seropositive sera. Serially diluted sera were tested for IL-6 bioactivity in a standard assay for IL-6. One unit of IL-6 bioactivity is defined as the activity inducing one-half maximal proliferation of the target B9 cells.

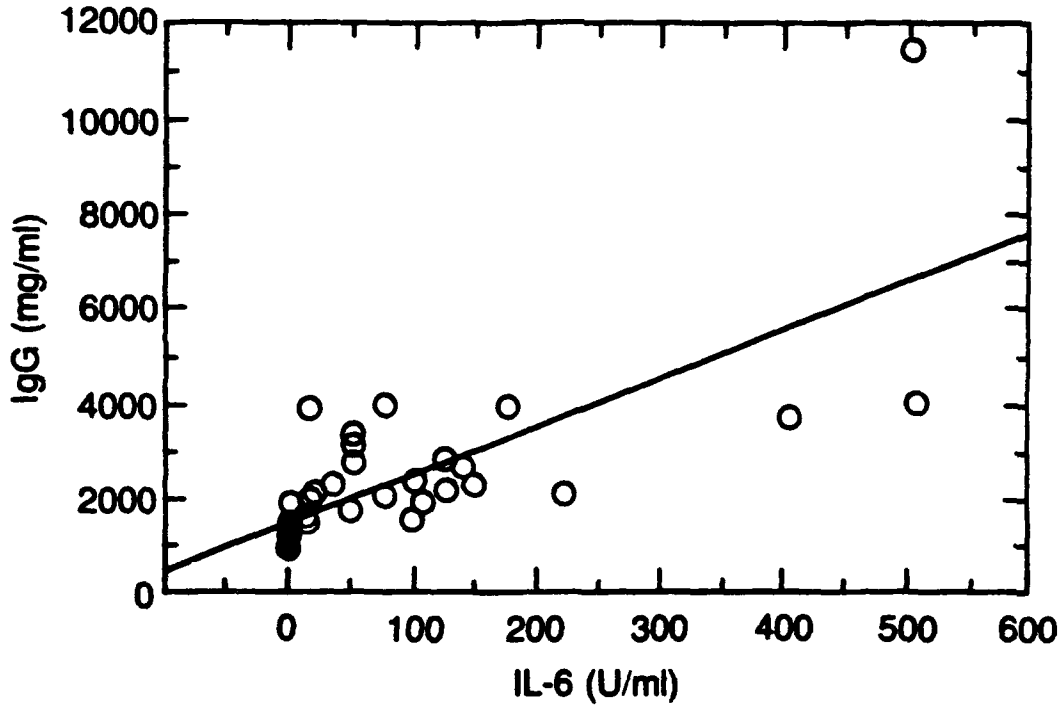


Fig. 13. Serum IgG levels correlate with serum IL-6 levels in HIV infected individuals. Serum IgG levels were plotted against serum IL-6 levels and correlation calculated by regression analysis, ($r = .74$, $P < .001$).

Table 1. Distribution of IL-6 bioactivity in the culture supernatants of EBV-immortalized cell lines

Cell line[a]	IL-6 bioactivity (U/ml)[b] in supernatant	
	Unfractionated	>5,000 mol wt
TB	1.6	0.83
La	14.4	14.0
TI	4.4	3.2
RY	0.5	1.2
Rb	17.5	3.35
3/22	9.7	6.1
TI	0.6	1.2

[a] Culture supernatants from the indicated cell lines (prepared as described in Materials and Methods) were size fractionated by ultrafiltration through a membrane with a 5,000-molecular-weight cutoff (YM5; Amicon). Unfractionated and size-fractionated supernatants were individually tested for IL-6 bioactivity, as described in the text.

[b] Units of activity per milliliter of supernatant. For concentrated (>5,000-molecular-weight) supernatants, the results are corrected for the concentration factor. No IL-6 bioactivity as found in supernatants with molecular weights lower than 5,000.

Table 2. Lactic acid induces proliferation in EBV-immortalized B cells

Lactic acid (mM)	Proliferation cpm/culture ($x10^3$)		
	Exp 1	Exp 2	Exp 3
5.0	29.1	5.1	25.5
2.5	29.6	4.6	23.8
1.25	13.6	3.5	14.7
0.6	5.9	2.0	12.3
0	3.4	0.4	8.8

The lymphoblastoid cell line VDS-O was cultured (2.5×10^3 cells/ml) for 3 days in RPMI 1640 medium supplemented with BSA (1 mg/ml) and transferrin (2.5 μg/ml) either alone or with lactic acid and at the indicated concentrations. Cultures were pulsed with ^3H thymidine during the final 18 hours of a 3 day culture.

TABLE 3

IL-6 secretion in lymphoblastoid cell lines

Cell line	Infecting virus	IL-6 activity* U/ml/48hr
VDS-0	None	18.4
VDS-0 1.3	pZip-Neo-SV(x)1	2.8
VDS-0 1.4	pZip-Neo-SV(x)1	3.75
VDS 1.5	pZip-Neo-SV(x)1	3.2
VDS-0 1	pZip-Neo-SV(x)1-IL6	7600
VDS-0 2	pZip-Neo-SV(x)1-IL6	826
VDS-0 3	pZip-Neo-SV(x)1-IL6	6750
TB	None	1.2
TB 2.2	pZip-Neo(x)1	0
TB 3.3	pZip-Neo(x)1	0
TB 4.4	pZip-Neo(x)1	0
TB 1	pZip-Neo-SV(x)1-IL6	40,562
TB 5	pZip-Neo-SV(x)1-IL6	16,750

*IL-6 activity was determined in a standard B9 culture assay and is expressed as units per ml of culture supernatant conditioned by 0.5×10^6 cells for 48 hr. Each data point corresponds to the mean of at least 3 determinations.

TABLE 4

Tumorigenicity of EBV-induced lymphoblastoid cell lines in athymic mice

Cell line	Tumor Incidence	Mean Time of First observation (days)	Mean maximum tumor size (mm^2)
VDS-0 parent	3/20	32	218
VDS-0 1.3 control	0/7	—	—
VDS-0 1.4 control	0/5	—	—
VDS-0 1.5 control	1/8	36	39
VDS-0 1 IL-6	9/15	21	42
VDS-0 2 IL-6	12/15	25	140
VDS-0 3 IL-6	12/15	23	168
TB parent	1/20	48	103
TB 2.2 control	0/6	—	—
TB 3.3 control	0/6	—	—
TB 4.4 control	0/8	—	—
TB 1 IL-6	9/15	33	27
TB 5 IL-6	9/15	27	45

Outbred four to six weeks old female athymic mice were inoculated subcutaneously in one site with 10^7 cells from the indicated cell lines and observed for 15 weeks for appearance of tumors > 5 mm^2 in surface area. Data are compiled from 4 separate experiments. Tumor incidence is expressed as the fraction of mice with tumor per number of mice injected. Tumor size is expressed in surface area and reflects the product of two-dimensional caliper measurements.

TABLE 5

Relationship between levels of IL-6 production and tumor development

Cell line	IL-6 level (U/ml)*	Tumor incidence‡	Mean time of first observation	Mean maximum size (mm^2)§
VDS-0 2	low (826)	12/15	35	140
VDS-0 1	intermediate (7,600)	9/15	21	42
TB 1	high (40,562)	9/15	33	27

* IL-6 levels are expressed as B9 U/ml supernatant conditioned by $0.5x10^6$ cells in 48 hr.

‡ Tumor incidence is expressed as number of animals with tumor per number of animals injected. Each animal received 10^7 cells subcutaneously in one site.

§ Tumor size is expressed in mm^2 and reflects the product of two-dimensional caliper measurements.

TABLE 6

IL-6 inhibits the activation of IL-2 stimulated killer cells

Exp	Culture* conditions	% SPECIFIC KILLING							
		VDS-0 Cell Targets				YAC Cell Targets			
		100:1	50:1	25:1	12:1	100:1	50:1	25:1	12:1
1	IL-2	81[i]	54	38	20	62	58	48	35
	IL-2+IL-6	28	26	16	7	40	37	28	26
2	IL-2	80	60	49	29	97	81	79	72
	IL-2+IL-6	39	29	5	4	63	61	48	28
3	IL-2	50	49	33	14	46	44	41	20
	IL-6	2	0	0	0	6	1	0	1
	IL-2+IL-6	15	14	3	5	28	23	29	16

* Fresh splenocytes from 4 to 6 weeks old nude mice were incubated ($2x10^6$ cells/ml, 2 ml/well) with either IL-2 alone (500 U/ml) or IL-6 alone (10^8 U/ml) or IL-2 (500 U/ml) plus IL-6 (10^8 U/ml) for 4 days in complete mouse cell culture medium. After incubation, the cells were tested for killing of ^{51}Cr-labeled VDS-0 or YAC-1 cell targets at the indicated ratios.

[i] Results of cytotoxicity assays are expressed as mean % specific lysis of triplicate culture. Standard deviations were within 15% of the mean.

Table 7. IL-6 Secretion in Normal Monocyte Cocultured with Mononuclear Cells from Normal or HIV-Seropositive Individuals

Cocultures	No.	p24 Levels (pg/mL)	IL-6 Activity (U/mL) Mean x/+ SEM
Normal (HIV-seronegative)	5	<10	6.7 x/+ 5
HIV seropositive	12	<10	5.1 x/+ 2.5
HIV seropositive	12	<250	48 x/+ 6.3

Cell-free culture supernatants of monocyte-enriched populations cocultured with either normal or HIV-seropositive PBMC were tested in parallel for the presence of the HIV-associated antigen p24 and for IL-6 bioactivity. p24 Levels were determined by ELISA; a culture was considered HIV-positive if p24 levels in the supernatants were greater than 250 pg/mL in 2 consecutive weeks. IL-6 levels (expressed in units per milliliter) were determined by a standard growth assay for B9 cells. Data shown reflect parallel determinations on day 84 of coculture or at an earlier time point for HIV-positive cultures (mean = 36 days). Culture medium used for virus isolation had a mean of 2 x/+ 1.3 U/mL of IL-6.

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Tosato, G., Miller, G., Marti, G., and Pike, S.E.: Accessory function of IL-1 and IL-6: Preferential costimulation of T4 positive lymphocytes. Blood 75:922-930, 1990.

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Tanner, J.E. and **Tosato, G.**: Impairment of natural killer functions by IL-6 increases lymphoblastoid cell tumorigenicity in athymic mice. J. Clin. Invest, in press.

List of Publications Submitted Resulting from Contract No. 87PP7855

Tanner, J.E. and **Tosato**, G.: Regulation of B cell growth and immunoglobulin gene transcription by interleukin 6, submitted.

Frugoni, P. and **Tosato**, G.: A mechanism of T cell regulation of Epstein-Barr virus latency, submitted.

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